MOLECULAR AND CELLULAR BIOLOGY, Sept. 1992, p. 3883–3889
0270-7306/92/093883-07$02.00/0

Vol. 12, No. 9

Roles of Insulinlike Growth Factor 1 (IGF-1) and the IGF-1 Receptor in Epidermal Growth Factor-Stimulated Growth of 3T3 Cells

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Received 14 April 1992/Returned for modification 13 May 1992/Accepted 9 June 1992

BALB/c3T3 cells are exquisitely growth regulated and require platelet-derived growth factor, epidermal growth factor (EGF), and insulinlike growth factor 1 (IGF-1) for growth. When BALB/c3T3 cells are transfected with plasmids constitutively expressing both EGF and the human IGF-1 receptor mRNAs, the cells are capable of growing in serum-free medium without the addition of any exogenous growth factor. These cells, called p5 cells, can grow for prolonged periods in serum-free medium. BALB/c3T3 cells transfected with only the IGF-1 receptor expression plasmid (p6 cells) do not grow in serum-free medium but do grow if IGF-1 (or insulin in supraphysiological concentrations) is added. p6 cells also grow in response to EGF, confirming that the combination of EGF and an overexpressed IGF-1 receptor is sufficient for the growth of 3T3 cells. We have found that in EGF-stimulated p6 cells there is an increase in the expression of IGF-1 mRNA, that IGF-1 is secreted into the medium, and that the growth of p5 cells and EGF-stimulated p6 cells is inhibited by exposure to antisense oligodeoxynucleotides to IGF-1 receptor RNA. Finally, while cells constitutively expressing both EGF and EGF receptor RNAs grow, albeit modestly, in serum-free medium, their growth is also inhibited by an antisense oligodeoxynucleotide to IGF-1 receptor RNA. In contrast, in cells overexpressing the IGF-1 receptor, IGF-1-mediated cell growth occurs independently of the platelet-derived growth factor and EGF receptors (Z. Pietrzkowski, R. Lammers, G. Carpenter, A. M. Soderquist, M. Limardo, P. D. Phillips, A. Ullrich, and R. Baserga, Cell Growth Differ. 3:199–205, 1992, and this paper). These data indicate that an important role for EGF is participation in the activation of an autocrine loop based on the IGF-1–IGF-1 receptor interaction, which is obligatory for the proliferation of 3T3 cells.

The growth of fibroblasts and fibroblastlike cells in culture is regulated by growth factors in the medium. In BALB/c3T3 cells, platelet-derived growth factor (PDGF) and platelet-poor plasma are both necessary for sustained growth (37, 38). Platelet-poor plasma can be replaced by epidermal growth factor (EGF) and insulinlike growth factor 1 (IGF-1) or by insulin at high concentrations (11, 22, 41). Other cell lines, such as, for instance, BHK cells (3) and WI-38 human diploid fibroblasts (31), also require more than one growth factor for optimal growth in culture. The inability of EGF or IGF-1 individually to stimulate fibroblast cell proliferation has been attributed to the low numbers of IGF-1 binding sites (1, 7, 33, 46). PDGF, by increasing the number of IGF-1 binding sites (6, 46), renders cells responsive to the stimulatory effect of IGF-1 alone.

In a previous study (33), we have shown that 3T3 cells constitutively overexpressing the human IGF-1 and IGF-1 receptor (IGF-1R) cDNAs grow in serum-free medium (SFM) without the addition of exogenous growth factors. Cells overexpressing only the IGF-1R cDNA (p6 cells) did not grow in SFM but grew vigorously after the addition of IGF-1. In that study, we also showed that, in these cells, IGF-1-mediated cell growth occurred in the absence of activation of either the EGF or the PDGF receptor.

In the experiments described below, we show that the growth-promoting effect of EGF on 3T3 cells requires the IGF-1–IGF-1R interaction even when the EGF receptor is overexpressed, while constitutive overexpression of the IGF-1R makes the cells capable of responding with growth to IGF-1 only, to the exclusion of the PDGF and EGF receptors.

MATERIALS AND METHODS

Plasmids. Cvn-IGF-1 receptor contains the full-length coding sequence of the human IGF-1R cDNA under the control of the simian virus 40 (SV40) promoter and the coding sequence of the Neo-R gene, also under the control of the SV40 promoter (20, 45). Cvn-IGF-1 contains a 600-bp *Xho*I-*Eco*RV cDNA fragment of human IGF-1 under the control of the SV40 early promoter, courtesy of Axel Ullrich (Max-Planck-Institut fur Biochemie, Martinsried bei Munchen, Germany). Cvn-EGF consists of synthetic human EGF cDNA in an expression vector under the control of the SV40 promoter (gift from A. Ullrich). The pXER plasmid contains the human EGF receptor cDNA under the control of the SV40 early promoter (gift from G. Gill and G. Rosenfeld, La Jolla, Calif.). pLHL4 was used to confer resistance to hygromycin (13). All Cvn plasmids included the Neo-R gene.

Cell lines. New cell lines derived from BALB/c3T3 cells (p5, p6, p12, and p17) were established by transfection or cotransfection (39) of the appropriate plasmids with selectable markers. BALB/c3T3 cells expressing a human IGF-1R sequence (Cvn-IGF-1R) under the control of the SV40 promoter are called p6 cells. p12 cells were established from p6 cells by cotransfection with pCvn-IGF-1 and pLHL4 (33). p5 cells also express three plasmids, pCvn-IGF-1R, pCvn-

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EGF, and pLHL4 (as a selectable marker), as do p17 cells, which express pCvn-EGF, pXER, and pLHL4. For selection, G418 was used at a concentration of 400 μg/ml and hygromycin was used at a concentration of 200 μg/ml.

Cell culture. All cell lines tested were passaged in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal bovine serum and 5% calf serum. To grow p6, p5, p12, and p17 cells in SFM, DMEM supplemented with bovine serum albumin (BSA) (0.7%) and $FeSO_4$ at a final concentration of 1 μM was used. Plating in serum-supplemented medium for 24 h was done to allow the cells to attach to the surface before being placed in SFM (33). For quiescence of BALB/c3T3 cells, instead of SFM, 0.5% calf serum in DMEM was used for 72 h. Unless otherwise stated, the concentrations of growth factors were as follows: IGF-1, 50 ng/ml (Bethesda Research Laboratories, Inc.), EGF, 20 ng/ml (GIBCO); and PDGF BB, 3 ng/ml (GIBCO).

Synthetic oligodeoxynucleotides. All oligonucleotides were synthesized on an Applied Biosystems, Inc., model 391 EP DNA synthesizer with β-cyanoethyl phosphoromidite chemistry. Oligonucleotides were added to cultures of 3T3-derived cell lines twice, the first time (40 μg/ml) 24 h after plating in SFM and the second time (20 μg/ml) the next day. 3T3 cells were also treated with oligonucleotides twice, the first time (40 μg/ml) 72 h after plating in 0.5% calf serum in DMEM and the second time 24 h later (20 μg/ml). Two hours later, the cells were stimulated to grow with PDGF BB plus EGF.

The antisense oligonucleotide to human IGF-1R RNA, 5′ TCC TCC GGA GCC AGA CTT, and the sense oligonucleotide, 5′ AAG TCT GGC TCC GGA GGA, correspond to codons 21 to 26 of the signal sequence of the subunit of IGF-1R preceding the proreceptor sequence (45). Another synthetic sense oligonucleotide, 5′ GAA AGG AAG CGG AGA GAT, and the antisense oligonucleotide 5′ ATC TCT CCG CTT CCT TTC correspond to nucleotides 2251 to 2269 of the putative precursor processing site (45).

Mouse antisense oligonucleotide to the EGF receptor. The antisense oligonucleotide 5′ GGC CAT TTT GGA GAA TTC and the sense oligonucleotide 5′ GAA TTC TCC AAA ATG GCC correspond to nucleotides 3100 to 3118 of the β domain (15).

^{125}I-IGF-1 binding. Recombinant human IGF-1 was from Amersham, Arlington Heights, Ill., and had a total activity of ~2,000 Ci/mmol. IGF-1 binding was carried out as previously described (32, 33).

RT-PCR of mouse IGF-1. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out as previously described (34). To generate a 267-bp fragment of mouse IGF-1 cDNA, 18-mers were used as amplimers. The 5′ amplimer ATG TCG TCT TCA CAC CTC corresponds to codons −22 to −17 of the signal peptide of the mouse liver IGF-1 sequence (2). The 3′ amplimer (in antisense orientation) AGG CTT CAG TGG GGC ACA corresponds to nucleotides 181 to 199. As a probe, we used an oligonucleotide whose sequence corresponds to nucleotides 105 to 123 of the same sequence. RNA was extracted by the method of Chomczynski and Sacchi (4). Labeling of probes and hybridization were carried out by standard methods (8, 43). RNA amounts were monitored with the 3A10 plasmid, whose insert is expressed constantly throughout the cell cycle (23).

IGF-1 radioimmunoassay. Conditioned medium containing 0.1% BSA–1 μM ferric sulfite was collected after stimulation with exogenous EGF (10 ng/ml) for 15 and 30 min and 1 and 6 h as appropriate. To remove IGF-1-binding proteins, 0.1 ml of conditioned medium was mixed with 900 μl of 1 M acetic acid and 5% BSA and loaded on SepPak C_{18} columns (Waters, Milford, Mass.). Before being loaded, the column was washed with 10 ml of methanol and then with 10 ml of H_2O.

After being loaded, the column was washed with 10 ml of 4% acetic acid and IGF-1 was eluted in 1 ml of 50% acetonitrile–4% acetic acid. After lyophilization, the sample was resuspended directly in 100 μl of radioimmunoassay buffer. The assay was performed according to the instructions of the kit's manufacturer (Amersham). IGF-1 radioimmunoassay was performed with a rabbit antiserum and a second antibody bound to magnetic beads (Amersham).

Phosphorylation of IGF-1R. Cells were stimulated with the ligand at 37°C. The cells were placed on ice and rinsed with cold Hanks buffered saline. Cells were lysed with lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10% glycerol, 1% Triton X-100, 100 mM NaF, 0.2 mM sodium orthovanadate, 10 mM sodium PP_i) containing 10 mM phenylmethylsulfonyl fluoride and 0.1 mg of aprotinin per ml. After a 3-min lysis at 4°C, the lysate was centrifuged for 2 min (4°C) to remove nuclei. The cleared lysate was transferred to a fresh tube, and 1 volume of HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, 10 mM NaF) was added. One microgram of monoclonal antibody to IGF-1R (Oncogene Sciences, Uniondale, N.Y.) was added. Anti-mouse immunoglobulin G–agarose conjugate (100 μg) (Sigma Immunochemicals, St. Louis, Mo.) was then added to immunoprecipitate the antibody-receptor complex. Antibody-antigen complexes were allowed to form for 2 h at 4°C and were then centrifuged at 4°C for 5 min. The complex was washed three times with HNTG, and 20 μl of Laemmli buffer (20% glycerol, 3% sodium dodecyl sulfate, 3% β-mercaptoethanol, 10 mM EDTA, 0.05% bromophenol blue) was added. Samples were boiled for 5 min, and proteins were separated on a 7% polyacrylamide gel. Proteins were then electroblotted onto a nitrocellulose filter. Phosphorylated proteins were detected by Western blot (immunoblot) analysis using standard techniques with an antiphosphotyrosine antibody (UBI, Saranac Lake, N.Y.). Bound phosphotyrosine antibodies were detected by using the ECL detection system from Amersham.

RESULTS

The previous finding (33) that p6 cells, 3T3 cells constitutively overexpressing a human IGF-1R cDNA, can grow in IGF-1 only and without the activation of the PDGF and EGF receptors prompted us to investigate the effects of other growth factors on these cells. Figure 1 shows that p6 cells in SFM respond to the addition of EGF with vigorous growth; in fact, they respond almost as well as when they are stimulated with IGF-1. Under these conditions, the parent cell line, BALB/c3T3, does not grow appreciably after addition of either EGF or IGF-1 (Fig. 1). p6 cells have five times the number of IGF-1 binding sites of 3T3 cells (33).

In a previous study (33), we had shown that 3T3 cells constitutively overexpressing both IGF-1 and IGF-1R cDNAs (p12 cells) grew in SFM without the addition of any exogenous growth factor. We transfected p6 cells (overexpressing only the IGF-1R) with an expression plasmid carrying a synthetic human EGF sequence. Several clones expressing the synthetic EGF RNA (not shown) were isolated, and all displayed behavior similar to that of clone p5,

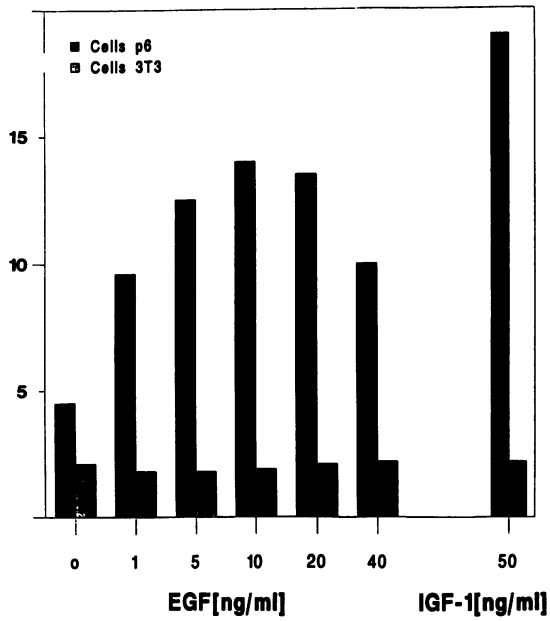

FIG. 1. Effect of EGF on the growth of 3T3 and p6 cells. Cells were plated at a concentration of 10^4 cells per cm^2 and were made quiescent as described in Materials and Methods. They were then stimulated with either EGF or IGF-1 at the concentrations indicated on the abscissa. The ordinate gives the cell concentrations (number of cells [10^4] per square centimeter) 48 h later.

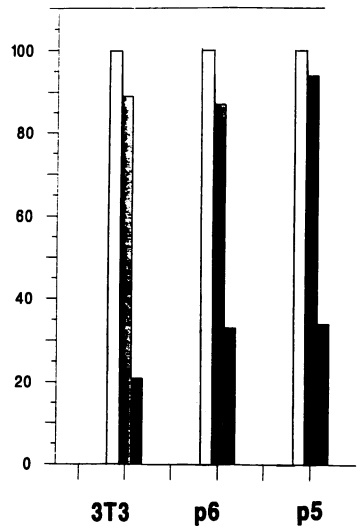

FIG. 3. Effect of antisense oligodeoxynucleotides to IGF-1R RNA on the growth of 3T3 cells and derivative cell lines. Cells were made quiescent (see Materials and Methods) and were then incubated with sense and antisense oligomers to IGF-1R RNA in SFM for 1 h before stimulation with EGF only (p6 cells), PDGF plus IGF-1 (3T3), or nothing (p5). The ordinate gives the percent increase in the number of cells after 48 h. Open bars, controls (no oligomers); striped bars, sense oligomer (40 μg/ml); closed bars, antisense oligomer to IGF-1R RNA (40 μg/ml).

which is illustrated in Fig. 2. Cells constitutively overexpressing both EGF and IGF-1R cDNAs grow very well in SFM, almost as well as p12 cells.

In the experiment whose results are shown in Fig. 1, we had found that 3T3 cells do not respond to EGF only, while p6 cells do. Presumably, the only difference between 3T3 and p6 cells is in the number of IGF-1 binding sites (see above). We therefore questioned whether an antisense oligodeoxynucleotide to IGF-1R RNA had an inhibitory effect on the growth of p5 cells (overexpressing EGF and IGF-1R) and on the EGF-mediated stimulation of growth in p6 cells. The results are shown in Fig. 3. An antisense oligodeoxynucleotide to IGF-1R RNA (but not a control oligomer) inhibits the growth of p5 cells, of EGF-stimulated p6 cells, and of 3T3 cells stimulated with both PDGF and EGF.

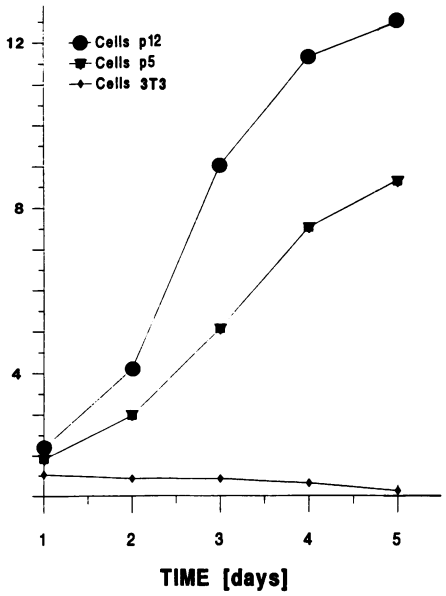

FIG. 2. Growth of 3T3 cells and derivative cell lines in SFM. Cells were plated as described in Materials and Methods; after 24 h, they were incubated in SFM (day 1) and the cell concentrations (number of cells [10^4] per square centimeter) (ordinate) at various intervals were determined. 3T3, BALB/c3T3 cells; p5, 3T3 cells stably transfected with EGF and IGF-1R expression plasmids; p12, 3T3 cells stably transfected with IGF-1 and IGF-1R expression plasmids.

Specificity of the antisense oligodeoxynucleotide to the IGF-1R RNA. To support the crucial point that the antisense oligomer to the IGF-1R RNA did inhibit the desired target, we performed two experiments. In the first, we determined the level of IGF-1R that is autophosphorylated by IGF-1 (see Materials and Methods). The results are shown in Fig. 4. Lane 1 is the control (no IGF-1 added). Lane 2 is the lysate from control cells, and lane 3 is the lysate from cells treated with the antisense oligomer to IGF-1R RNA for 48 h; both are shown 15 min after the addition of IGF-1. The antisense oligomer causes a 60% decrease (as determined by densitometry) in the amount of IGF-1R that can be autophospho-

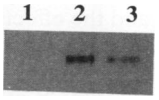

FIG. 4. Effect of an antisense oligodeoxynucleotide to the IGF-1R RNA on the levels of the receptor. The amount of autophosphorylated IGF-1R was determined as described in Materials and Methods for p6 cells incubated in SFM for 48 h. Lane 1, negative control, no IGF-1 added; lanes 2 and 3, cells grown with sense (lane 2) or antisense (lane 3) oligodeoxynucleotides to IGF-1R RNA, 15 min after addition of IGF-1 (25 ng/ml).

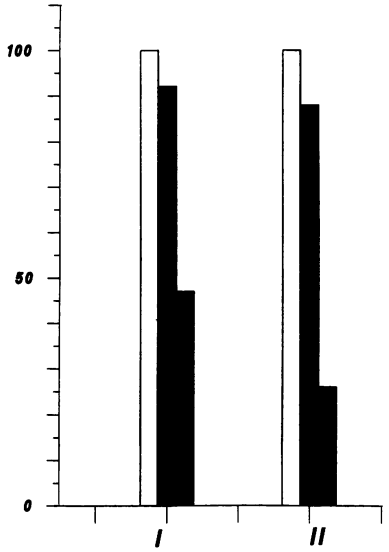

FIG. 5. Two different antisense oligodeoxynucleotides to IGF-1R RNA inhibit IGF-1-mediated cell growth. Quiescent p6 cells were incubated with sense or antisense oligomers (40 μg/ml) for 1 h and then stimulated with IGF-1 (50 ng/ml). The ordinate gives the percent increase in the number of cells after 48 h. I, original oligomer (signal peptide); II, second oligomer (see Materials and Methods). Open bars, no oligomers (control); checked bars, sense oligodeoxynucleotides; closed bars, antisense oligodeoxynucleotides.

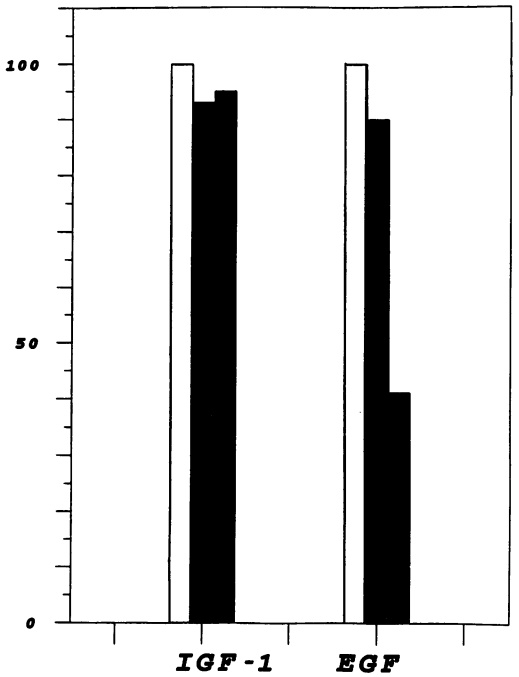

FIG. 6. An antisense oligomer to the EGF receptor RNA does not inhibit IGF-1-mediated stimulation of p6 cells but does inhibit EGF-mediated stimulation. Quiescent p6 cells were stimulated with either IGF-1 (50 ng/ml) or EGF (20 ng/ml). The ordinate gives the percent increase in the number of cells after 48 h. Open bars, control (no oligomers); checked bars, sense oligomer (40 μg/ml); closed bars, antisense oligomer (40 μg/ml) to the EGF receptor RNA.

rylated. In the second experiment, we tried a second antisense sequence, targeted to a different region of the human IGF-1R RNA (see Materials and Methods). Its effect on the growth of p6 cells is shown in Fig. 5. The second antisense oligomer to IGF-1R RNA is even more effective than the original one in inhibiting growth of p6 cells. These two experiments strongly indicate that our antisense oligodeoxynucleotides specifically inhibit the expression of the IGF-1R.

An antisense oligomer to the EGF receptor does not inhibit IGF-1-mediated cell growth. We have shown that an antisense oligomer to the IGF-1R RNA inhibits both IGF-1- and EGF-mediated stimulation of p6 cells (33) (Fig. 3), which constitutively overexpress the IGF-1R. We next questioned what the effect of an antisense oligomer to the EGF receptor RNA (mouse sequence) would be. We tested this antisense oligomer in p6 cells stimulated by either IGF-1 or EGF. The results of a typical experiment are shown in Fig. 6. An antisense oligomer to the EGF receptor RNA has no effect on the growth of IGF-1-stimulated p6 cells, confirming our previous findings that, under these conditions, the EGF receptor is excluded (33). The same antisense oligomer, however, inhibits the growth of the same cells stimulated by EGF, indicating both the necessity of the EGF receptor under these conditions and the effectiveness of the antisense oligomer on the correct target. Inhibition of EGF-mediated growth by an antisense RNA to the EGF receptor has been previously reported by Moroni et al. (26).

EGF increases the levels of IGF-1 and IGF-1 mRNA. The experiments described above suggest that EGF stimulation of p6 cells requires a functional IGF-1R; it is reasonable to assume that EGF may actually induce the production of IGF-1. Figure 7 shows the results of an RT-PCR of IGF-1 mRNA after p6 cells have been stimulated with EGF (20 ng/ml). The levels of IGF-1 mRNA increase 10-fold (as determined by densitometry) in the first 2 h after EGF stimulation of p6 cells, then decrease, and then increase again. Controls with the 3A10 probe (23) indicated similar amounts of RNA in each reaction (not shown). IGF-1 is secreted into the medium when p6 cells (incubated in SFM for 48 h) are stimulated with EGF. As determined by radioimmunoassay, the amounts of IGF-1 (nanograms per milliliter per 1.5×10^6 cells) in the medium of EGF-stimulated p6 cells were 3.2 at 2 h after stimulation and 6.9 at 6 h after stimulation. Untreated cells had nondetectable levels of IGF-1. An antisense oligodeoxynucleotide to IGF-1 RNA inhibited EGF-mediated stimulation of p6 cells (not shown).

The IGF-R is activated in cells constitutively expressing IGF-1 or EGF. To confirm that EGF induces growth in p5 (or p6) cells through the activation of the IGF-1R, we measured the autophosphorylation of the IGF-1R under various con-

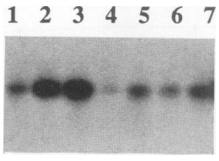

FIG. 7. Increased expression of IGF-1 mRNA after EGF stimulation of p6 cells. Quiescent p6 cells were stimulated with EGF (20 ng/ml), RNA was extracted at different intervals, and the amount of IGF-1 mRNA was determined by RT-PCR (20 cycles). Lanes (from left to right) show results at 0, 1, 2, 4, 6, 8, and 24 h after EGF stimulation.

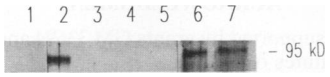

FIG. 8. Autophosphorylation of the IGF-1R under different conditions. Autophosphorylation in SFM was determined by immunoprecipitation with an antibody to the IGF-1R and staining with an antiphosphotyrosine antibody (see Materials and Methods). Only the B chain (95 kDa) is stained. Lanes: 1, unstimulated p6 cells; 2, p6 cells stimulated with IGF-1 for 15 min; 3 to 5, p6 cells, 5, 15, and 60 min, respectively, after addition of EGF (20 ng/ml); 6, p5 cells; 7, p12 cells. The last two were exponentially growing in SFM, and no IGF-1 was added.

ditions, using immunoprecipitation and staining with a phosphotyrosine antibody (see Materials and Methods). The results are shown in Fig. 8. No signal is detectable in unstimulated p6 cells in SFM (lane 1). Autophosphorylation of the IGF-1R is easily detectable in the same cells 15 min after addition of IGF-1 (lane 2); EGF (20 ng/ml) does not cause autophosphorylation of IGF-1R within the first 60 min (lanes 3 to 5), indicating that there is no direct interaction of EGF with the IGF-1R. However, a strong positive signal is detected in both p12 cells (overexpressing IGF-1 and IGF-1R) (lane 7) and p5 cells (constitutively overexpressing EGF and IGF-1R) (lane 6) growing in SFM in the absence of addition of exogenous IGF-1. These experiments indicate that the IGF-1R is constitutively autophosphorylated in cells growing in SFM and constantly secreting either IGF-1 or EGF.

An antisense oligodeoxynucleotide to IGF-1R RNA inhibits the growth of cells constitutively expressing EGF and EGF receptor RNAs. Since 3T3 cells constitutively expressing both the IGF-1 and IGF-1R RNAs grow in SFM (33), and since overexpression of single growth factors' receptors can make cells capable of growth in response to the ligand only (17, 24, 35, 36), we have questioned whether the constitutive expression of EGF and EGF receptor RNAs could also make 3T3 cells capable of growing in SFM. We transfected BI-4 cells, previously established in our laboratory from 3T3 cells (11) and expressing a synthetic human EGF gene, with an EGF receptor expression plasmid (see Materials and Methods) plus a selectable marker, generating clones designated p17 that constitutively express both EGF and EGF receptor RNAs (data not shown). p17 cells (or at least the several clones tested) grew in SFM (Fig. 9), albeit not as vigorously as p12 cells, i.e., the 3T3 cells constitutively expressing the IGF-1 and IGF-1R RNAs (33). The growth of p17 clones was inhibited by an antisense oligodeoxynucleotide to the IGF-1R RNA (but not by a sense oligomer [Fig. 9]). On day 4, the inhibition of growth was almost 60%.

A legitimate question can be raised at this point: why EGF cannot stimulate 3T3 cells and yet p17 cells can grow in SFM, albeit requiring the IGF-1R. The response of 3T3 cells to IGF-1 depends on the number of IGF-1 receptors (5–7). Experiments with SFM show that, indeed, the number of IGF-1 binding sites is increased in p17 cells, although not as much as in p6 cells. As determined by the method of Phillips et al. (32), the numbers of IGF-1 binding sites per cell for each cell line tested were as follows: 3T3 cells, 8,000; p6 cells, 43,000; and p17 cells, 25,000.

DISCUSSION

The interaction of IGF-1 with its receptor seems to play a major role in the control of both normal and abnormal

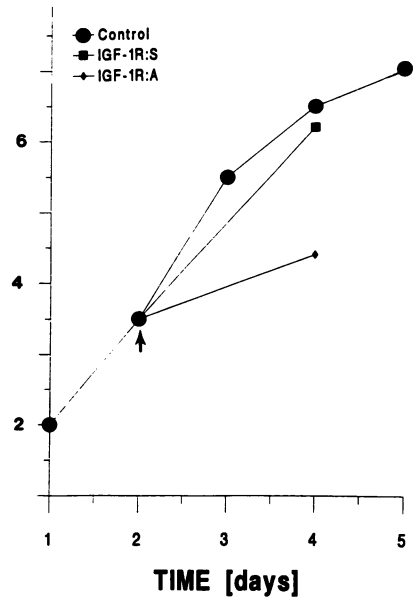

FIG. 9. Growth of p17 cells in SFM. p17 cells are 3T3 cells stably transfected with EGF and EGF receptor expression plasmids. They were plated at 10^4 cells per cm^2, and the ordinate gives the cell concentrations (number of cells [10^4] per square centimeter) at the days indicated on the abscissa. Control, p17 cells; IGF-1R:S and IGF-1R:A, sense and antisense oligodeoxynucleotides to the IGF-1R RNA, respectively (both at 40 μg/ml).

growth. Although IGF-1 alone does not stimulate the growth of fibroblastlike cells such as mouse 3T3 cells or human WI-38 diploid fibroblasts, it is required, together with PDGF and/or EGF, for optimal growth (11, 22, 37, 38, 41). Some hemopoietic cell lines grow in and require IGF-1 or high concentrations of insulin for growth (14, 18, 19, 21, 29); it is generally accepted that, at high concentrations, insulin acts through the IGF-1R (9). In our laboratory, using an antisense oligodeoxynucleotide to the IGF-1R RNA, we have recently demonstrated that a functional IGF-1R is necessary for growth of T lymphocytes stimulated by phytohemagglutinin and interleukin 2 (33a) and of serum-stimulated human diploid fibroblasts (38a). Goldring and Goldring (12), in a review, list several cell types which require IGF-1 for growth, including keratinocytes, smooth muscle cells, chondrocytes, osteoblasts, and others. IGF-1 stimulates meiosis in *Xenopus* oocytes as effectively as progesterone (14), plays a major role in development (46, 47), and regulates the mRNA levels of cdc2 (42). The transforming gene of the avian sarcoma virus UR-2 (v-*ros*) is homologous equally to IGF-1 and to insulin receptors, which are in fact more similar to each other (45) than they are to v-*ros*. In addition, IGF-1Rs have been reported to be abundant in a variety of human tumors (10, 21, 25, 27, 30) and are induced by estrogens in breast cancer cells (28, 30, 40).

Recently, we have shown that 3T3 cells constitutively overexpressing the IGF-1R RNA (p6 cells) grow in SFM supplemented with IGF-1 alone (33). Under these conditions, the number of IGF-1 binding sites increased from 8,000 per cell in 3T3 cells to 46,000 per cell in the p6 cell line. This increase was sufficient to make 3T3 cells capable of a growth response to the same concentrations of IGF-1 that do not have any effect on the parent cell line, BALB/c3T3. The IGF-1-mediated stimulation of growth of p6 cells occurred in

the absence of activation of either the PDGF receptor or the EGF receptor. The exclusion of the EGF receptor under these conditions was confirmed in this study by the fact that an antisense oligomer to EGF receptor RNA did not inhibit IGF-1-mediated growth of p6 cells (Fig. 6), although the same antisense oligomer inhibited EGF-stimulated growth.

We are aware that overexpression of a growth factor receptor almost invariably allows a cell to grow in the presence of the ligand only (see, for instance, references 17, 24, 35, and 36 and Fig. 9). Indeed, McCubrey et al. (24) even showed that an overexpressed IGF-1R can abrogate an interleukin 3 requirement in hemopoietic cells. However, the novelty here is that cells with the overexpressed IGF-1R do not need the EGF (or PDGF) receptor for growth in IGF-1, while p6 cells stimulated by EGF or cells overexpressing both EGF and the EGF receptor RNA still need a functional IGF-1R for mitogenic stimulation. This seems to give the IGF-1–IGF-1R interaction a privileged status in the control of cell proliferation, at least in 3T3 cells.

Indeed, our experiments show that (i) EGF does not stimulate 3T3 cells but does stimulate 3T3 cells overexpressing the IGF-1R (p6 cells) (Fig. 1 and 2); (ii) an antisense oligodeoxynucleotide to the IGF-1R RNA inhibits IGF-1-stimulated p6 cells, p5 cells (overexpressing EGF and IGF-1R), 3T3 cells stimulated by PDGF plus EGF (Fig. 3), and p17 cells, overexpressing both EGF and EGF receptor RNAs (Fig. 9); and (iii) in contrast, an antisense oligomer effective against the EGF receptor does not inhibit IGF-1 stimulation of p6 cells. EGF per se does not activate the IGF-1R (Fig. 8); in fact, EGF is often used as the negative (noncompetitive) control in IGF-1 binding assays (32, 33).

The fact that EGF stimulates p6 (but not 3T3) cells suggests that EGF may increase IGF-1 production. This is in fact the case: levels of both IGF-1 mRNA and IGF-1 in the medium are demonstrably increased after EGF stimulation of p6 cells. This is not to say that the only function of EGF is to induce IGF-1, a finding already reported by Clemmons et al. (5–7), but it is obviously an important function. With a relatively low number of IGF-1 binding sites (3T3 cells), EGF, although inducing IGF-1, is not able to stimulate growth, but with a higher number of IGF-1 binding sites (p6 and p12 cells) the increased production of IGF-1 results in growth. There is a discrepancy in the fact that EGF cannot stimulate 3T3 cells yet can make p17 cells grow in SFM, although still requiring the IGF-1R. It seems that added EGF cannot induce a sufficient number of IGF-1 binding sites but can do so if steadily supplied into the medium. This discrepancy requires further investigation.

In conclusion, our experiments again emphasize the pivotal role of the IGF-1R in the growth of 3T3 cells, even when the stimulation of cellular proliferation begins with the activation of the EGF receptor by EGF. Indeed, we can say that an important role of EGF is to participate in the activation of an autocrine loop based on the IGF-1–IGF-1R interaction, which is obligatory for the proliferation of 3T3 cells. Since PDGF is known to increase the number of IGF-1 binding sites (5), one could easily visualize a situation in which PDGF and EGF cooperate in making the cells responsive to IGF-1. Finally, as it becomes increasingly evident that the activation of the IGF-1R by its ligand is required by several cell types for growth (for a review, see reference 1), and since the proto-oncogene c-*myb* increases the expression of both IGF-1 and IGF-1R (34, 44), one can legitimately inquire how many other growth factors (including hemopoietic ones [24]) and proto-oncogenes may act through this pathway.

ACKNOWLEDGMENT

This work was supported by grants GM 33694 and AG 00378 from the National Institutes of Health.

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